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Research paper

The Effect of Ethanol Extract of *Calotropis gigantea* Root in Increasing the Level of IFN-γ and the Expression of Caspase 3 on Mice (*Mus musculus L.*) with Fibrosarcoma

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Abstract

Calotropis gigantea is one of the traditional medicine especially used for cancer treatment. This study aimed to evaluate the effect of ethanol extract of C. gigantea root in increasing the level of IFN-γ and the expression of Caspase 3 as a scientific evidence. The ethanol extract at dosage of 50, 100 and 150 mg/kg bw were given daily for several weeks on the mice induced by 7,12-dimethylbenz(α) anthracene (DMBA). The result revealed that the dosage of 150 mg/kg bw significantly could increase the level of IFN-γ and the expression of Caspase 3. In addition, the dosage 150 mg/kg bw also increases the body weight especially after induction of DMBA. This result indicate that the active ingredients of C. gigantea root have a function as immunomodulator agent to increase immune system, initiate apoptosis process and make proliferation disfunction by increasing IFN-γ and Caspase 3. This finding showed that C gigantea useful for the treatment of fibrosarcoma better than methotrexate as anticancer medicine standard.

Keywords: *Calotropis gigantea, fibrosarcoma, DMBA, Caspase 3, IFN-γ*

1. Introduction

Calotropis gigantea (*C. gigantea*) or biduri (Indonesia) belonging to family Asclepiadaceae, is commonly found in tropical region and most abundant in Indonesia. Different organs of *C. gigantea* have been reported to be used for treatment of diaphoretic, anxiety, pain, purgative, analgesic, digestive, tonic, diarrhea, and pregnancy interceptive properties (Araújo *et al.*, 2013; Habib & Karim, 2013). Several active ingredients have also been reported to be isolated from *C. gigantea* such a and b-calotropeols, C31 and C33 hydrocarbons, b-amyrin and its isovalerate, mixture of triterpene, sterols, fatty acids, giganteol, glycosides, calotropin, uscharin, calotoxin, gigantin and uscharidin (Habib & Karim, 2013; Kadiyala *et al.*, 2013).

By this study we found that bioactive, antioxidant properties and biological activity of *C. gigantea* could function as immuno-modulator and therefore increase the immune system of the body. Polyphenol compounds contained in *C. gigantea* root are known to act as immuno-modulator which effectively stimulate IFN-γ (You *et al.*, 2013). IFN-γ is the primer cytokinin which activates $CD8^+$ on tumor cells. It is also very important and stimulating agent of immuno-surveillence component such as NK cell, cytotoxicity of T cell ($CD8^+$) and macrophag, which contributes in killing and apoptosis process of cancer cells (Morgado *et al.*, 2016). In addition, IFN-γ could also increase the activation of Caspase 3, an intracelullarly cytokin protease group that is an important component to response to apoptosis (Schleicher *et al.*, 2016). In this case, Caspase 3 is one of caspase effectors which contributes in proteolitic activation during the apoptosis process (Hensley *et al.*, 2013; Sinha *et al.*, 2013).

This study was aimed to determine the effect of ethanol extract of *C. gigantea* root in increasing the level of IFN-γ, expression of Caspase 3, and body weight of fibrosarcoma mice induced by DMBA. This study could be used as a baseline for developing ethanol extract of *C. gigantea* as functional traditional medicine especially to increase the immunity of the body as adjuvant therapy on fibrosarcoma cancer.

2. Material and Methods

2.1. Ethanol extraction of C. gigantea root

The procedure for the extraction of the root of collected C. gigantea referred the procedure described by Muchtaromah et al., (2011 and 2016) was used for extraction. The collected roots of C. gigantea were dried in circulating air oven (± 40 ºC), crushed and macerated until exhaustion in 70% ethanol solution at ratio of 1:5 (w/v) replaced every 72 h. The final extract (clear filtrate) was concentrated in a rotary evaporator under the reduced pressure at temperature of 60°C and subsequently lyophilized, covered by aluminum foil and kept at 4°C until used.

2.2. Animals

Mice were obtained from Laboratory of Animal Physiology, Department of Biology, Faculty of Science and Technology, State Islamic University of Malang. They were kept under standard environmental conditions (12 h dark/light cycle) and temperature (25 °C). Mice feed and water were given to the experiment animals ad libitum.
Induction of experimental fibrosarcoma
Fibrosarcoma was induced by DMBA with dosage of 25 mg/kg bw. The induction was performed by subcutaneous technique twice a week for around 6 weeks. On week 6 of DMBA induction, the animals were feed by ethanol extract of C. gigantea each day for 2 weeks.

3. Experimental design

In the experiment, the animals were randomly divided into 6 groups with 5 replications. Group 1 (C-) negative control or without induction of DMBA and C. gigantea therapy, group 2 (C+) positive control consisted of rats induced by DMBA without C. gigantea therapy, group 3, 4 and 5 (T1, T2, T3: DMBA-induced mice treated with ethanol extract C. gigantea of 50, 100 and 150 mg/kg bw/day for 2 weeks, respectively), group 6 (T4) DMBA-induced mice treated with 2.5 mg/kg bw of anticancer medicine, methotrexate, twice a week.

3.1. Determination of IFN-γ by ELISA method

The ELISA assay to measure IFN-γ was performed in 96-well vinyl plates (Costar 2595). The method and the specificity of the antisera referred to Schleicher et al. (2016). Each well was coated with 150 µl of a solution containing 1.5 µg/ml MD2 antibodies in a 0.05 M carbonate buffer (pH 9.6) for 3 h at 37°C. Then, each plate was rinsed with PBS containing 0.05% Tween 20 for five times. Samples of rHu lFN-y (Biogen, Geneva) or dilutions of culture supernatants were added to the wells. The plates were incubated for 1 h at 37 °C and subsequently rinsed several times. To each well 100 µl of rabbit anti- rHu lFN-y solution (diluted 1/800) in PBS containing 0.05% Tween 20 and 1% BSA were added. Then, to each plate, 100 µl of goat antirabbit IgG HRP (diluted 1/1000) in PBS containing 0.05% BSA was added. The normal mouse serum (NMS, diluted 1/100) was added to each plate containing solution in order to block affinity for mouse immunoglobulins, and incubated for 3 h at 37 °C.

3.2. Analysis of Caspase 3 Expression

The procedure to analyze caspase 3 expression described by Krajewska et al., (1997) with some slight modification was used. The in vivo patterns of Caspase-3 gene expression were determined using an immunohistochemical approach. Full-thickness skin and subcutaneous tissue samples were embedded and divided in several section. Sections were deparafinized, rehydrated, and equilibrated in wash buffer solution (0.05 M Tris-HCl, 0.1% Tween 20, and 0.3 M NaCl) for 30 min at room temperature. Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol for 30 min at room temperature. 5% normal rabbit serum was used to block excess proteins and then rinsed in wash buffer solution for 30 min at room temperature. The primary antibodies were diluted 1:1000 in Tris-buffered saline (TBS) in a humidified chamber equilibrated with phosphate-buffered saline (PBS) for 1 h at room temperature. A catalyzed signal amplification (CSA) kit (Dako) containing biotinylated rabbit anti-mouse IgG and streptavidinperoxide complex was used for antibody staining.
The immunohistochemical staining was performed on one or more sections per sample, and several randomly selected fields per each section were examined and observed under the microscope (400 x). According to the percentage of caspase - positive cells, immunostaining results were semiquantitatively scored based on spot with chocolate color as follows: score 0, when absent; score 1, when 1%-20% of chocolate color in the cells or in the field were very less viscos; score 2, when 21%-40% of chocolate color in the cells or in the field were less viscos; score 3, when 41%-60% of chocolate color in the cells or in the field were a bit viscos; score 4, when 61%-80% of chocolate color in the cells or in the field were viscos; and score 5, when 81%-100% of chocolate color in the cells or in the field were very viscos.

3.3. Statistical analysis

The effect of ethanol extract of C. gigantea root in increasing the level of IFN-γ and the expression of Caspase 3 was analyzed by one way analysis of variance (ANOVA). Post Hoc Turkey HSD was used for statistical comparison between control and various treated groups. Statistical significance was accepted at the $P < 0.05$ values.

4. Results

4.1. The effect of ethanol extract of C. gigantea root on body weight and IFN-γ level

The observation on the body weight was performed done from the first week to measure the early step of carcinogenesis and continued until week 10. Figure 1 shows the development of the body weight over time and it indicate the significant difference between the inductions of DMBA (C+, T1, T2, T3 and T4) with negative control or normal condition in the experimental groups. Therapy of ethanol extract of C. gigantea was given orally as long as 2 weeks after the induction DMBA twice a week, 6 weeks could significantly increase the body weight of mice. T3 has the highest body weight increase as compared to the other treatment groups.

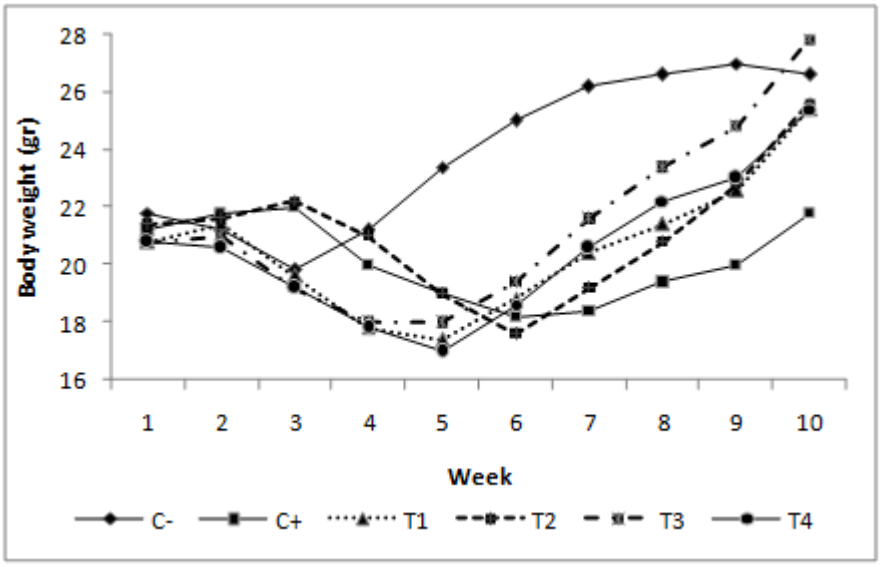

Fig 1: Effect of ethanol extract of the root of *C. gigantea* on body mass over 10 weeks. Perioda 1st week acclimation, 2nd - 7th weeks induction by DMBA, 8th week adaptation phase, 9th - 10th weeks extraction treatment by *C. gigantea* root. (C-) Negative control; (C+) Positive control; (T1) dosage of *C. gigantea* 50 mg/kg bw; (T2) 100 mg/kg bw; (T3) 150 mg/kg bw and (T4) Methotrexat.

Treatment of DMBA treated mice with different dosage of ethanol extract of *C. gigantea* root significantly effects the average level of IFN-γ. At a dosage of 150 mg/kg bw the highest increase of IFN-γ could be measured as compare to other treatments including treatment of anticancer medicine Methotrexat (Table 1).

Table 1: The averages of IFN- γ level on mice with fibrosarcoma with and without treatment by *C. gigantea* root

Group	Average ± SD (pg/ml)
C- (Negative control)	105 ± 44,3c
C+ (Positive control)	48,7 ±25,7a
T1 (50 mg/kg bw)	83,7 ± 41,5b
T2 (100 mg/kg bw)	114 ± 53,1c
T3 (150 mg/kg bw)	178,6 ± 79,2e
T4 (Methotrexat)	146,3 ± 86,6d

* Different notation indicates a significant different

4.2. The effect of ethanol extract of C. gigantea root on caspase 3 level

We also performed experiment to determine the effect of ethanol extract of C. gigantea root with different dosage on the averages of caspase 3 expression level. In this study, we use immuno-histochemistry on subcutaneous slides by using antibody caspase 3, in which cell expressing caspase 3 have cytoplasmic showing chocolate color. The result revealed that cell expressing caspase 3 were found in all treatment groups including the control (Fig.2A and 2B). However, an immuno-ratio analysis and followed by ANOVA analysis revealed that at a dosage of 150 mg/kg bw (T3) the highest caspase 3 expression level could be determined as compared to other treatments (Table 2).

Table 2: The averages (in) of caspase 3 expression level of mice with fibrosarcoma

Group	Average ± SD (%)
C- (Negative control)	79.92 ± 23.20d
C+ (Positive control)	31.85 ± 26.18a
T1 (50 mg/kg bw)	45.85 ± 18.50ab
T2 (100 mg/kg bw)	60.32 ± 13.2bc
T3 (150 mg/kg bw)	79.05 ± 12.05d
T4 (Methotrexat)	75.0 ± 22.13c

* Different notation indicates a significant different

5. Discussion and Conclusion

The treatment of mice by DMBA leads to carcinogenesis process. DMBA is a strong carcinogen which mainly causes fibrosarcoma cancer. In this study, all mice treated by DMBA showed fibrosarcoma cancer, characterized by several nodules, hair loss, and the decreases of body weight. C. gigantea root could led to increase the fibrosarcoma mice body weight, compared to positive control or to mice without the treatment by C. gigantea root. Body weight increase could be antioxidant content of C. gigantea. You et al., (2013) reported that phytochemistry content of plants shows the selective ability to kill cancer cell either by inhibiting apoptosis process in vitro and in vivo, or inhibiting cancer angiogenesis and metastasis. Alkaloid, as an antioxidants in the root of C. gigantea, is known to be responsible for inhibiting of the development of cancer cells. Therefore, the increasing of body weight after treatment by C. gigantea could be expected by the result of antioxidant role in inhibiting the development of fibrosarcoma on mice.

Cancer cell is known as non self which is antigenic on human immune system and it causes cellular and humoral response. To inhibit the development of cancer cells, the humoral immunity has the smallest role than cellular immunity, but it still makes an antibody on tumor antigen. In addition, interferon could increase or inhibit the function of cells. The main inhibition role is inhibiting the growth of normal cell and neoplastic cell. IFN-γ could increase the ability of macrophage to kill bacteria and protozoa by macrophage activation (Morgado

et al., 2016). In this study, we found that after the induction of C. gigantea root, the carcinogenesis process was inhibited. It is also indicated by the role IFN-γ, in which all treatment induced by C. gigantea could increase the level of IFN-γ. In line with this finding, Kadiyala et al., (2013) reported that polyphenol, one of antioxidant compound found in C. gigantea, has been known to have an antioxidant, antiinflammation and anticancer effect.

The mechanism by which polyphenol function as anticancer was previously reported by inhibiting the secretion of IL-10 and enhancing the secretion of IL-12. The later, IL-12 will enhance the secretion of IFN-γ (Kadiyala *et al.*, 2013). Another component having similar role as polyphenol is triterpenoid (*Di-(2-ethylhexyl) Phthalate* and *Anhydrosophoradiol-3-acetate*) (You *et al.*, 2013). These chemical antioxidant are also found in *C. gigantea* (Kadiyala *et al.*, 2013).

IFN-γ is cytokine group having an activity as antivirus, immuno-modulation, and anti-proliferative. This protein is synthesized by cell as a response to foreign induction such as virus, antigen, foreign nucleic acid, etc. IFN-γ is commonly used as therapy for tumor, cancer, and blood dysfunction. IFN-γ inhibits the replication of virus not only in the cell producing IFN-γ, but also in the tissue (Vassalli, 1992).

The study also performed an experiment to assess the effect of ethanol extract of C. gigantea root on the average of caspase 3 expression level. The result revealed that the induction of C. gigantea significantly affected the caspase 3 expression level on mice with fibrosarcoma. Caspase 3 is a protein which contributes to initiate the apoptosis process by intrinsic or extrinsic mechanism (Hensley et al., 2013).

In this study, the extract of C. gigantea increases the apoptosis activation of caspase 3. Caspase 3 (ACA) is an enzyme which contributes for the breaking of protein by protease group, which causes cellular apoptosis. Caspase also contributes in initiation phase of apoptosis. ACA inhibits the inflammation process by reducing the concentration of Nitric oxide (NO) and Cyclooxygenase-2 (COX)-2 in the cell. Inflammation process will cause protein damage which finally leads to DNA damage and mutation, and then induce tumor cell to become more active or more dangerous (proliferation dysfunction). In addition, ACA also inhibit the development of NF-κB (nuclear factor-kappa B), in which the activation of NF-κB could surprise the apoptosis and made proliferation dysfunction (Hensley et al., 2013; Sinha et al., 2013).

The increasing of caspase 3 expression and causing apoptosis on treatment group were due to flavonoid which is known as stimulant in the production of IFN-γ in immunosit population (Kadiyala et al., 2013). This IFN-γ is very important to enhance the CTL's and NK cell activation on the regulation of immune system on cancer cell (Morgado et al., 2016). If CTL's and NK cell is more active, the killing of tumor cell is also more active than usual, or apoptosis on tumor cell is more enhancive (Schleicher et al., 2016). Apoptosis could occur due to the activation of caspase enzyme, and the activation of this enzyme could be facilitated by several pathway system in the body such as T-cell Receptor (TCR) or activation of granzyme which enter into the cell through pore forming factors perforin (Hensley et al., 2013).

In conclusion, this study showed that different dosage of ethanol extract of C. gigantea could significantly increase the level of IFN-γ, the expression of Caspase 3 and the body weight of fibrosarcoma mice. This finding result in new herbal medicine that could be used to treat fibrosarcoma disease better than methotrexate as anticancer medicine standard.

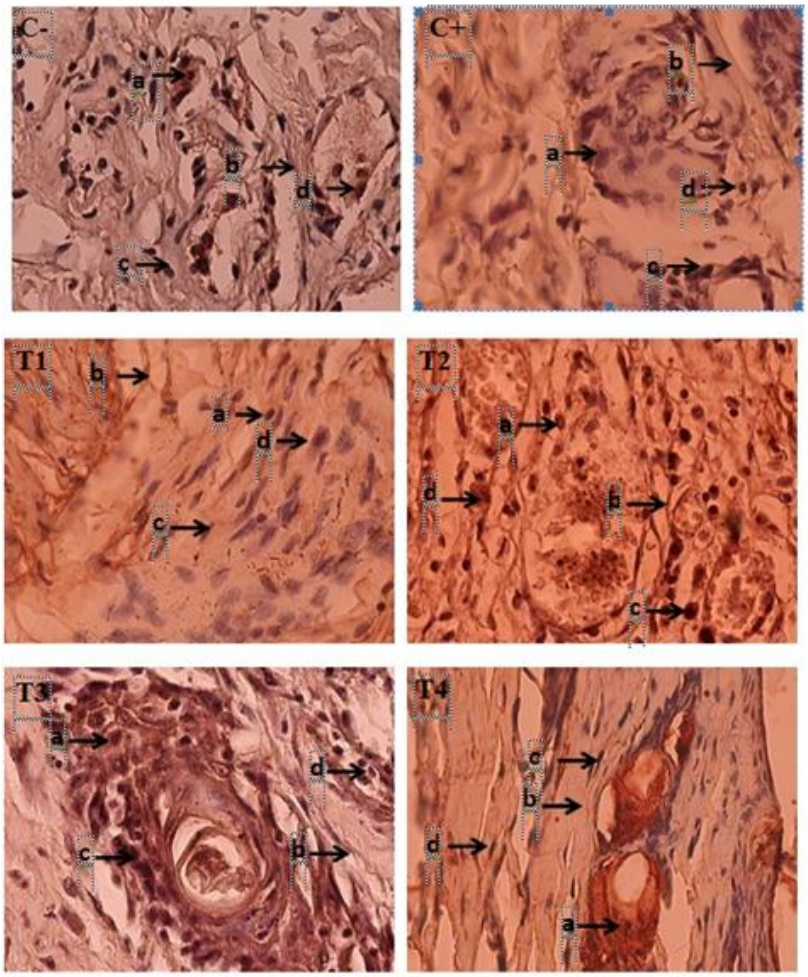

Fig. 2A: Coloring by immuno-histochemistry on subcutaneous tissue (400x). Expression level of caspase 3 was shown by chocolate color. a: nucleus, b: collagen fiber, c: fibroblast, d: cytoplasm.

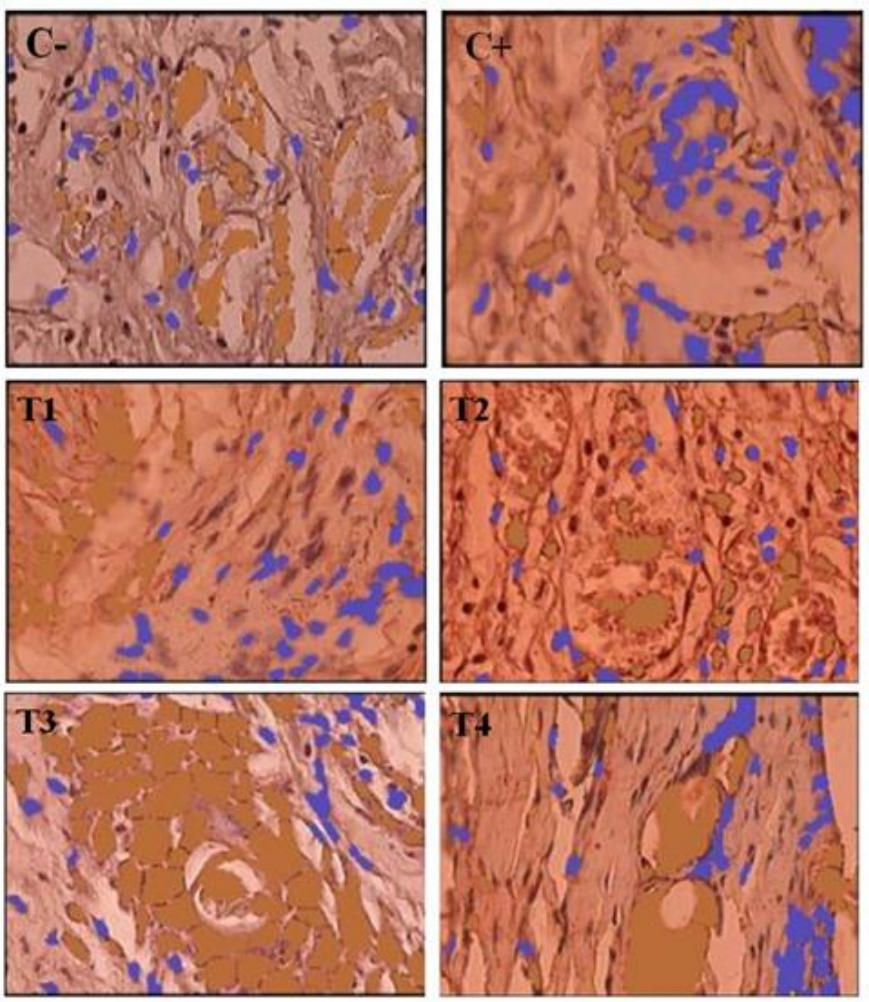

Fig. 2B: The width of caspase 3 expression level imaged by immune-ratio technique. Note: (C-) 79.92 %, (C+) 31.85 %,(T1) 45.85 %, (T2) 60.32 %, (T3) 79.05 %, (T4) 75%. Expression level of caspase 3 was shown by chocolate color ( ).

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